Final Report for AOARD Grant FA2386-10-1-4033 "**Biological and Nano-technological Applications of Artificial DNAs Made Exclusively of Nonnatutal C-Nucleosides with Four Types of Nonnatural Bases**"

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Abstract: We newly developed artificial nucleobases, G*, C*, D*, X^p*, D^p*, and T^p*, in addition to the original ones, A*, T*, iG*, and iC*. The structures of the new bases were based on detailed estimation for the thermal stabilities of the hetero duplexes that consist of an original artificial-DNA oligomer and a natural single-stranded DNA (ss-DNA). These new bases were successfully connected to our alkynyldeoxyribose skeleton, yielding the corresponding novel artificial nucleosides. Some of them could be oligomerized by means of DNA auto synthesizer to afford new nonnatural ss-DNAs. Furthermore, preliminary application of the artificial DNAs to an enzymatic reaction was fortunately succeeded. Nonnatural T* nucleoside was prosperously converted to the corresponding T* nucleotide with a naturally existing enzyme, human thymidine kinase 1 (TK1).

Introduction: We recently developed a new class of artificial DNA, which can be formed only from artificial DNA strands containing only nonnatural nucleoside residues possessing nonnatural four types of bases (A*, T*, iG*, and iC*) through nonnatural C-glycoside linkers.[1]   The hybridization occurred spontaneously and sequence selectively, and the resulting right-handed duplexes have net thermal stabilities very close to those of natural duplexes in spite of their differences for the geometries and hydrogen-bonding patters (Figure 1). These findings provide a further proof that a genetic phosphodiester-based molecular framework is not limited to the natural DNA one and can be extensible to many types of nonbiological entities. The artificial DNA exclusiveluy consists of nonnatural and chemically stable parts except for phosphodiester units. In addition, the helical structures and the thermal stabilities of the duplex formation are very close to those of natural DNA duplexes. Therefore, we consider that the artificial DNA can be applied to various types of natural enzymatic systems such as phospholyration, ligation, and template-directed replication, etc. If these processes will be successful, the artificial DNAs would have impacted widespread researches majoring in biological and material sciences. In the research duration of 1 year, the following two subjects are to be mission statements: **(1) structural extension of such artificial DNAs** and **(2) preliminary applications of the artificial DNAs to enzymatic reaction**.
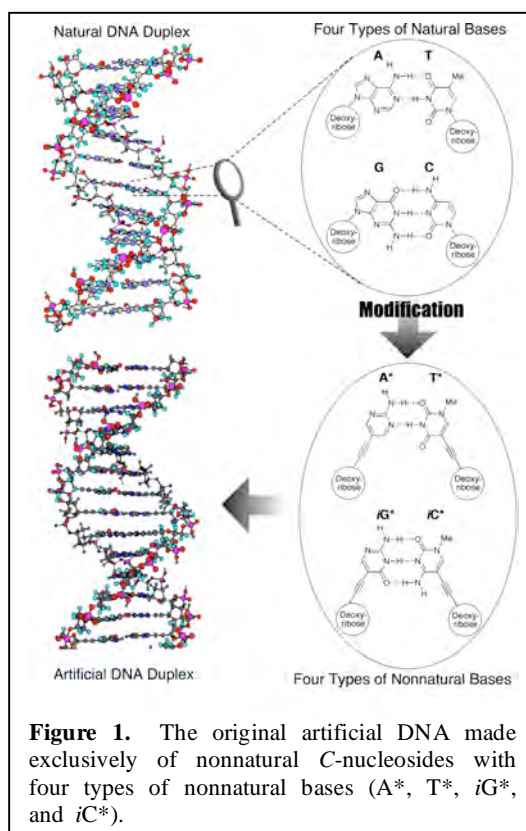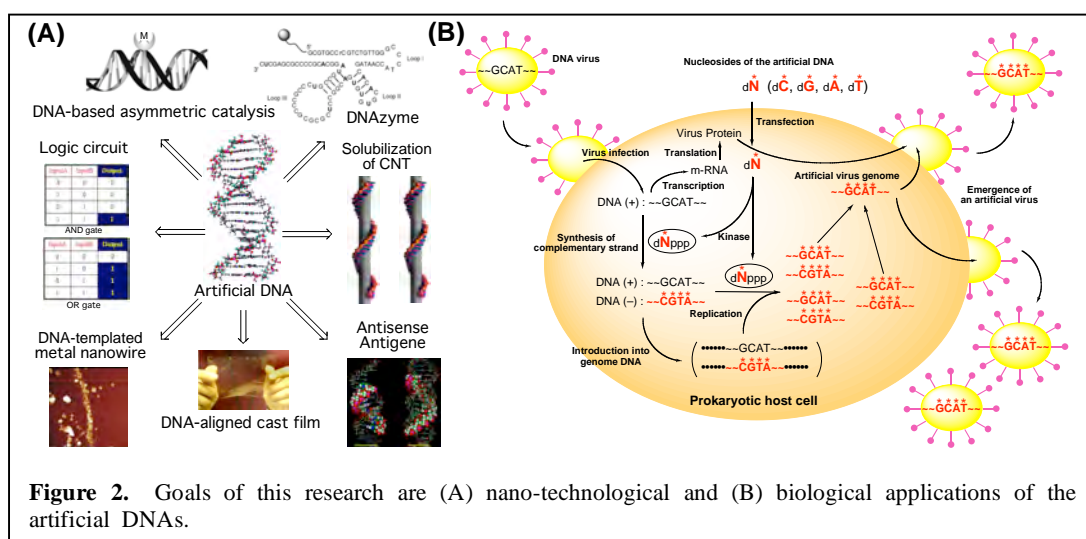

Figure 1. The original artificial DNA made exclusively of nonnatural C-nucleosides with four types of nonnatural bases (A*, T*, iG*, and iC*).

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14. ABSTRACT **This is the final report of a project on a new class of artificial DNA, which can be formed only from artificial DNA strands containing only non-natural nucleoside residues possessing non-natural four types of bases through non-natural C-glycoside linkers.**			
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After these, the artificial DNAs will be placed on a schedule in various types of biological and nanotechnological applications. The artificial DNA has inherent advantages of chemical stability and structural versatility, which enables the nonnatural DNA to be feasible functional nano-materials (Figure 2A). By use of the artificial DNA instead of natural DNA, we can create new types of nano-materials such as DNA-based asymmetric catalysis, solubilization materials for carbon nanotubes (CNT), DNA-templated metal nanowires, and DNA-aligned cast films. Furthermore, the artificial DNA will be attempted to develop new DNAzymes and antigene/antisense oligomers.

Figure 2B shows another biological approach, creation of artificial viruses possessing the artificial DNAs as a gene. By transfecting the nonnatural nucleosides into the host cell, a salvage pathway may work, which produces the artificial DNA oligomers of virus genomes. After amplification of the artificial virus DNAs, virus capsid incorporates the nonnatural genome DNAs, which may emerge a new artificial virus. If both of the virulence and temperateness of the original virus may not be observed, which indicates the inhibition of the proliferation of the infected DNA virus with the nonnatural nucleosides. In such a case, the artificial DNA may have a potential to be an antiviral agents based on lethal mutagenesis of viruses to error catastrophe.

Figure 2. Goals of this research are (A) nano-technological and (B) biological applications of the artificial DNAs.

Experiment:

Synthesis of Artificial DNA Oligomers. The artificial DNA oligomers were synthesized by use of the artificial phosphoramidites on an Applied Biosystems 392 synthesizer using standard β-cyanoethylphosphoramidite chemistry with the coupling reaction time of 15 min. The solid support (Universal Support II®), which allows for 3' placement of nonnatural nucleosides, was purchased from Glen Research. After automated synthesis, the oligomers were removed from the solid support with 2 M ammonia methanol solution at 30 °C for 30 min and deprotected with concentrated NH_4OH at 40 °C for 8 h. The oligomers were then purified by reverse-phase HPLC using a CHEMCOBOND 5-ODS-H column (10 x 150 mm) with an eluent of 5 mM ammonium formate and the following CH_3CN percentages of linear gradient (0–30 min, 0–15%) at a flow rate of 3.0 mL/min.

MALDI-TOF Mass Measurements. MALDI-TOF mass spectra were recorded on a Bruker-Daltonics-Autoflex mass spectrometer operating in the positive ion mode with 3-hydroxypicolinic acid as a matrix. $d(G^*)_8$: calcd for MH^+, $C_{88}H_{97}N_{24}O_{46}P_7$: 2444.43; found 2444.64, $d(C^*)_8$: calcd for MH^+, $C_{96}H_{113}N_{24}O_{46}P_7$: 2556.55; found 2556.28, $d(A^*)_{16}$: calcd for MH^+, $C_{176}H_{193}N_{48}O_{78}P_{15}$: 4693.88; found 4694.84, $d(T^*)_{16}$: calcd for MH^+, $C_{192}H_{209}N_{32}O_{110}P_{15}$: 5189.79; found 5191.35, $d(X^{p}*)_{12}$: calcd for MH^+, $C_{132}H_{146}N_{36}O_{70}P_{11}$: 3695.61; found 3695.97.

UV and T_m Measurements. UV-vis spectra and T_m melting curves (1.0 °C/1.0 min) were obtained by JASCO V-560 UV/VIS spectrophotometer with a peltier and a temperature controller in a temperature range from 0 to 90 °C. The T_m values were determined from the maxima of the first

derivatives of the melting curves measured in a buffer solution: 10 mM Hepes (pH 7.0), 10 mM MgCl$_2$, 100 mM NaCl. Errors were estimated at ± 1.0 °C. Concentrations of the solutions containing artificial DNAs were determined based on the molar extinction coefficients at 260 nm (ε_{260}) of the artificial nucleoside monomers.[1]
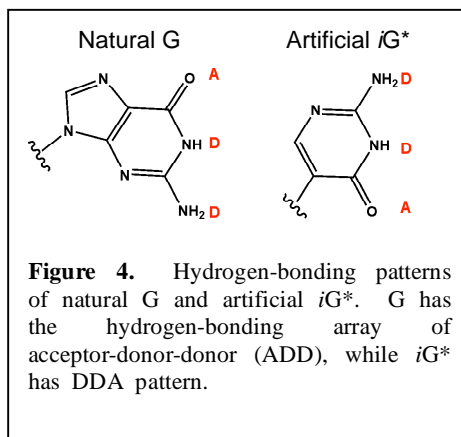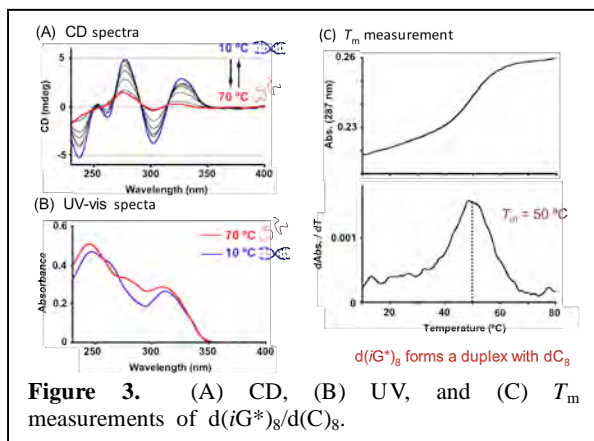
CD Measurements. CD spectra were recorded using a JASCO-J-720WI spectropolarimeter with a temperature controller at 0, 10, 20, 30, 40, 50, 60, and 70 °C in a buffer solution: 10 mM Hepes (pH 7.0), 10 mM MgCl$_2$, 100 mM NaCl.

Titration Experiments. Titration curves for artificial DNAs were obtained by monitoring a specified wavelength of CD or UV. In the case for d(A*)$_{16}$ versus dT$_{16}$, 3.0 mL of a d(A*)$_{16}$ solution (1.0 µM with 10 mM Hepes (pH 7.0), 10 mM MgCl$_2$, and 100 mM NaCl) was prepared, and CD measurement of the solution was carried out at 10 °C using a quartz cell of 1 cm pathlength. Separately, 1.0 mL of a dT$_{16}$ solution (100 µM in the same buffer) was then prepared, and 2.0 µL of the dT$_{16}$ solution (0.2 equivalent against the d(A*)$_{16}$) was added to the d(A*)$_{16}$ solution in the quartz cell. The mixed solution was stirred for 15 min at 10 °C, and then CD measurement was performed. A series of the operations were repeated for all the ratios of [dT$_{16}$]/[d(A*)$_{16}$] = 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, and 3.0. The normalized CD intensities at 315 nm were plotted against [dT$_{16}$]/[d(A*)$_{16}$] (Figure 6D).
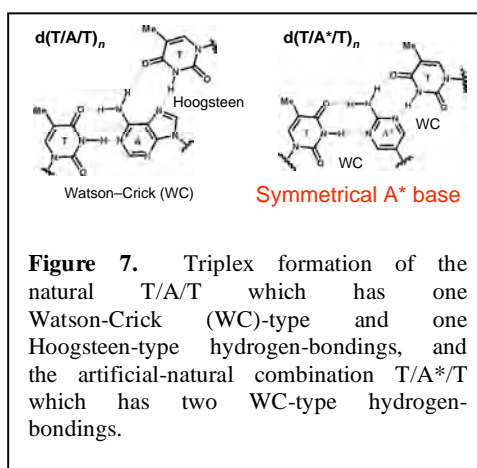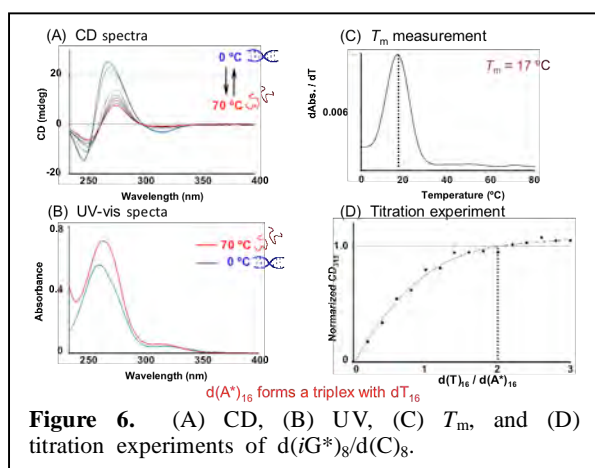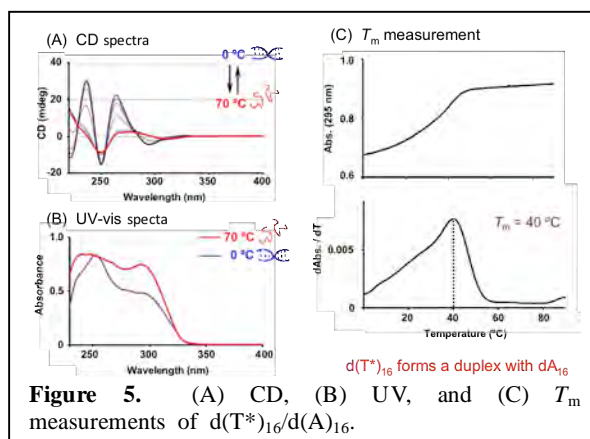
Results and Discussion:

(0)   Basic evaluation of artificial–natural hetero duplexes

Before structural choice of new artificial DNAs, thermal stabilities of the duplex formation were evaluated in detail for hetero duplexes that consist of the original artificial DNA strand (A*, T*, iG*, and iC*) and a natural one.   The hetero duplex formation has a potential toward various types of biological and nanotechnological applications in its own right. We prepared artificial and natural DNA oligomers, d(iG*)$_8$ and dC$_8$, respectively. Figure 3 displays the charts of CD, UV, and T_m measurements for a 1:1 mixed solution of the two oligomers. Significant Cotton effects of the mixture at room temperature began to weaken upon heating with isodichroic points and finally almost disappeared at 70 °C, accompanied with hyperchromicity at 287 nm in the UV-vis spectra. A cooperative, sigmoidal transition was clearly observed at 50 °C for the mixture by the T_m measurement. These observations indeed suggest the formation of a complementary hydrogen-bonding duplex of d(iG*/C)$_8$.   The relative stabilities of the duplexes decreased in the following order: d(iG*/iC*)$_8$, (T_m = 59 °C) > d(G/C)$_8$, (T_m = 55 °C) > d(iG*/C)$_8$, (T_m = 50 °C).[1]   Unfortunately, the lowest stability for the artificial–natural combination was observed.   The main reason of this trend is considered that the nonnatural iG* base has a reversed hydrogen-bonding pattern relative to that of the natural G base (Figure 4), which causes the hetero duplex to deform in the higher-order structures compared with artificial homo and natural homo helices.   Thus, this observation decided us to design new artificial bases, G* and C*, which have the same hydrogen-bonding patterns with natural G and C.

**Figure 3.** (A) CD, (B) UV, and (C) T_m measurements of d(iG*)$_8$/d(C)$_8$.

Figure 4. Hydrogen-bonding patterns of natural G and artificial iG*. G has the hydrogen-bonding array of acceptor-donor-donor (ADD), while iG* has DDA pattern.

Next, we examined another sets of artificial–natural combinations, d(T*)$_{16}$/dA$_{16}$ and d(A*)$_{16}$/dT$_{16}$. Duplex formation was confirmed for d(T*)$_{16}$/dA$_{16}$ by means of CD, UV, and T_m measurements (Figure 5) as the same trends for the duplex formation of d(iG*/C)$_8$ in Figure 3. Interestingly, homooligomers composed of nonnatural A* and natural T found to behave somewhat unlikely to the corresponding natural A–T combination. The titration experiment between d(A*)$_{16}$ and dT$_{16}$ was conducted on the basis of CD spectra monitored by 315 nm at 0 °C after annealing process (Figure 6D). The 1:2 (A*:T) stoichiometry for this

Figure 5. (A) CD, (B) UV, and (C) T_m measurements of d(T*)$_{16}$/d(A)$_{16}$.

complexation was confirmed by an apparent saturation at the molar ratio of T/A* = 2. Moreover, the first derivative profile of the melting curve had only one maximum point at 17 °C, indicating a strongly cooperative two-state transition (Figure 6C). These observations unambiguously indicate the exclusive formation of d(T/A*/T)$_{16}$-type triplex without d(A*/T)$_{16}$-type duplex, which is the same tendency with the spontaneous triplex formation of the artificial A*/T* combination.[1] This strong trend for the formation of triplexes over duplexes may due to the symmetry of A* along the axis from the amino nitrogen to the carbon connected to the acetylene linker (Figure 7). This observation also inspired us to design a new artificial base, improved A*, which has a dissymmetrical structure for its hydrogen-bonding sites.


Figure 6. (A) CD, (B) UV, (C) T_m, and (D) titration experiments of d(iG*)$_8$/d(C)$_8$.

Figure 7. Triplex formation of the natural T/A/T which has one Watson-Crick (WC)-type and one Hoogsteen-type hydrogen-bondings, and the artificial-natural combination T/A*/T which has two WC-type hydrogen-bondings.

(1) Structural extension of artificial DNAs

Based on the above evaluation for the hetero duplexes, we designed new artificial bases, G*, C* and D* (dissymmetric A*) as shown in Figure 8. The hydrogen-bonding patterns of G* and C* are the same to those of the natural G and C, respectively. D*, as a nonnatural nucleobase for dissymmetric-type of A*, has DAD-type hydrogen-bonding array which is complementary to nonnatural T* and natural T bases. Synthetic routes of the new base derivatives (G*-Br, C*-Br, and D*-I) and the corresponding nucleosides are displayed in Schemes 1–3. For G*-Br (Scheme 1), starting from 2-aminopyridine, the amino group was protected with *tert*-butyloyl group. Then bromination of the pyridine moiety and deprotection of the amino group were performed. N-oxidation of the pyridine nitrogen and the following

**Figure 8.** Newly designed artificial base pairs.
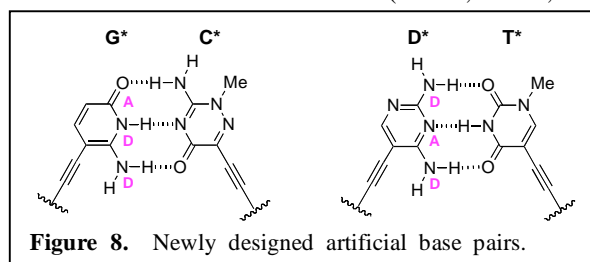
rearrangement afforded a 6-amino-2-pyrimidinone derivative. G*-Br was obtained by deprotection of the *O*-acetyl and the *N*-acetyl groups. Protected G* nucleoside was synthesized by an HMDS-assisted palladium-coupling reaction[2] (Sonogashira reaction) between G*-Br and an ethynyldeoxyribose derivative[3] in good yield. For C*-Br (Scheme 2), reaction of aminoguanidine and trichloroformaldehyde in HCl aq. gave aminoguanidine monoacetic acid, which heterocyclized by further heating to afford a 1,3,4-triazine derivative. Following bromination and *N*-methylation were performed to obtain C*-Br. C* nucleoside was also furnished by an HMDS-assisted Sonogashira reaction and following deprotection reactions in a moderate yield in 3 steps. Iodination of 2,4-diaminopyridine gave D*-I, and subsequent Sonogashira palladium-coupling reaction was carried out to give D* nucleoside. Appropriate protections on the nucleobases and ordinal phosphoramidation reactions proceeded successfully for these three nucleosides. Oligomerization of these phosphoramidite derivatives is now underway.
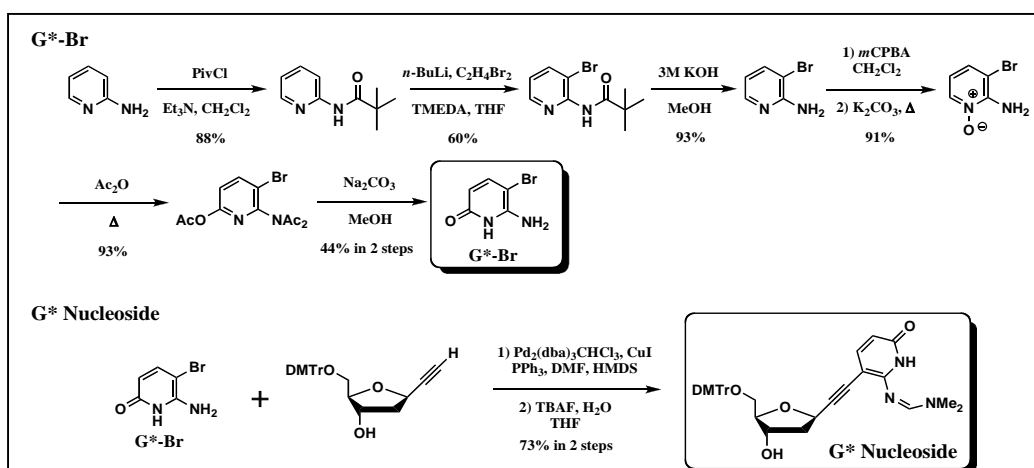
Scheme 1. Synthesis of G*-Br and G* nucleoside.
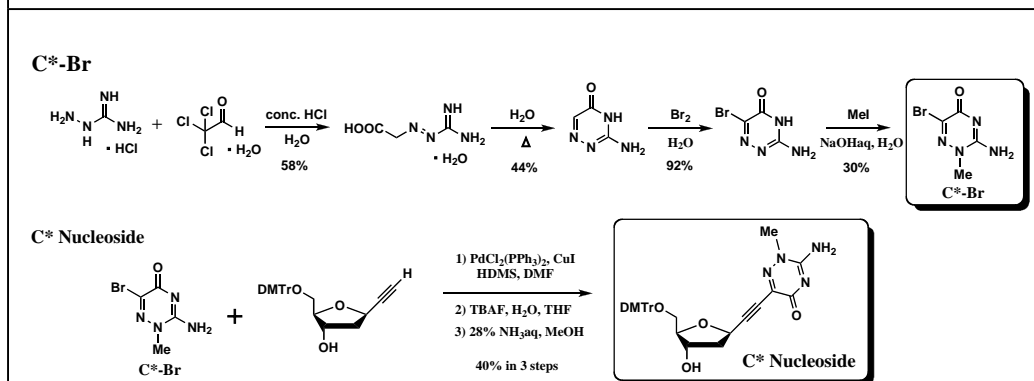
Scheme 2. Synthesis of C*-Br and C* nucleoside.
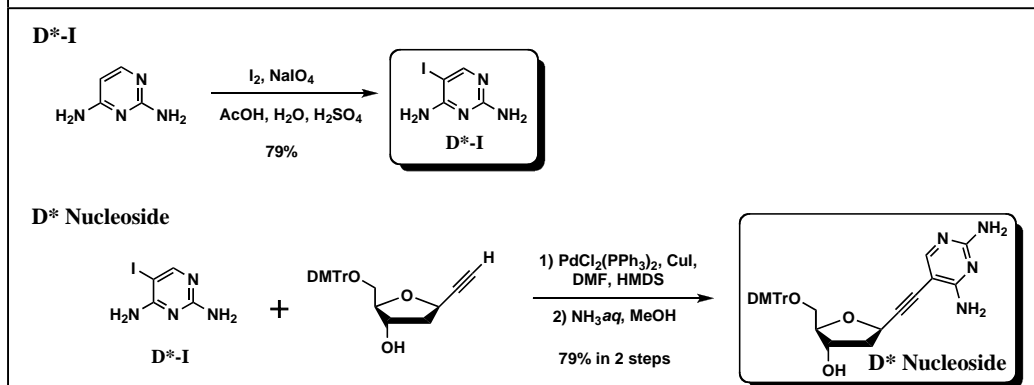
Scheme 3. Synthesis of D*-I and D* nucleoside.

Another set of genetic letters, X^p*, D^p*, and T^p* is newly designed for a construction of duplexes with a three-bases genetic system, in which the acetylenes connect the bases to the deoxyribose in a "*para*" fashion (Figure 9). The set comprises only three letters, X^p*, D^p*, and T^p* because the X^p* base of the 2-amino-3*H*-pyrimidine-4-one structure can tautomerize to its 1*H* form, and the resulting two tautomers can self-associate.[4] Iodinated nucleobases were synthesized for X^p*, D^p*, and T^p* (Scheme 4). For X^p*-I, halogen exchange reaction of an amino-dichloroprymidine derivative was conducted to obtain 2-amino-4,6-diiodopyrimidine. This compound was treated in an alkali aqueous solution to give a nonprotected X^p* skeleton, and following protection of the amino group afforded X^p*-I. D^p*-I was successfully synthesized by iodination of 2,4-diamino-6-chloropyrimidine. For T^p*-I, treatment of 2,4,6-trichloropyrimidine in alkali aqueous solution gave a chlorouracil derivative. The chlorine was exchanged to iodine, and then *N*-methylation of the uracil nitrogen afforded T^p*-I. Sonogashira palladium-coupling reaction was then carried out for these three nucleobases with the ethynyldeoxiribose unit. The following appropriate deprotection yielded the artificial nucleosides bearing X^p*, D^p*, and T^p* in good yields.

Subsequently, the X^p* nucleoside was converted into the corresponding phosphoramidite derivative. Single-stranded artificial DNA oligomer, $d(X^p*)_{12}$, could be obtained by means of DNA synthesizer (Figure 10). Detailed investigation for the properties of the new artificial DNAs is now in progress.
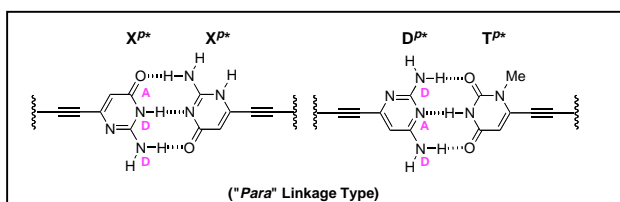

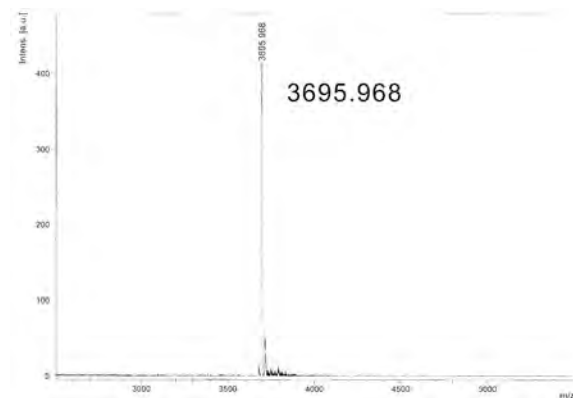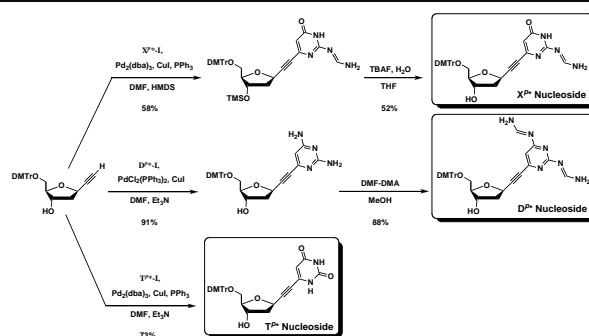**Figure 9.** Newly designed three-bases genetic system.

Scheme 4. Synthesis of X^p*-I, D^p*-I, and T^p*-I.

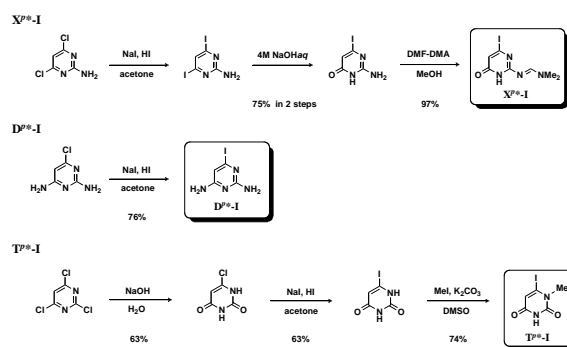**Scheme 5.** Synthesis of X^p*, D^p*, and T^p* nucleosides.

Figure 10. MALDI-TOF mass spectrum for $d(X^p*)_{12}$.

(2) Preliminary applications of the artificial DNAs to enzymatic reaction

As the simplest attempt to enzymatic reaction with the artificial DNAs, we selected mono-phosphorylation of the T* nucleoside with human thymidine kinase 1, TK1 (Scheme 6). Indeed, synthesis of nucleotides from nucleosides is very important in living cell as seen in the early steps of salvage pathways. In advance, we chemically synthesized the T* monophosphate (dT*MP) as a reference for HPLC analysis (Scheme 7) to determine the retention time of dT*MP and the HPLC condition for monitoring the progress of the enzymatic phosphorylation of dT*. The purified dT*MP was characterized by ^{1}H NMR and ESI mass measurements. In reverse-phase HPLC analysis, ATP, ADP, dT*MP, and dT* displayed their retention times at 1.9, 3.0, 12.2, and 15.1 min., respectively, in the analysis condition of a CHEMCOBOND 5-ODS-H column with an eluent of 10 mM ammonium formate and the following CH_3CN percentages of linear gradient (0–40 min, 0–30%) at a flow rate of 1.0 mL/min. Figure 11 shows an HPLC chart of the enzymatic reaction (Scheme 6) at 37 ºC after 18 hours. Although several unknown peaks were appeared and the starting dT* had been remained, enzymatic production of dT*MP was distinctly observed at the retention time of 12.2 min. Preparative purification and subsequent ESI mass analysis were carried out for this peak, indicating the certain identification of dT*MP (Figure 12). Therefore, appropriate progress of the enzymatic reaction was observed for the nonnatural T* nucleoside with TK1. Improvement of the reaction yields and utilization of different enzymes are now in progress.

In conclusion, we newly designed and synthesized several nonnatural bases, G*, C*, D*, X^{p}*, D^{p}*, and T^{p}*, and the corresponding artificial nucleosides. For the X^{p}*, phosphoramidation and oligomerization by DNA auto-synthesizer could be successively carried out. As the most importance in the duration of 1 year, we got a cue for enzymatic applications of our artificial DNAs as seen in the progress for enzymatic reaction of T* nucleoside with TK1. In near future, these results may lead satisfaction of biological and nanotechnological applications of our artificial DNAs.

Enzymatic Phosphorylation with Thymidine Kinase 1 (TK1)
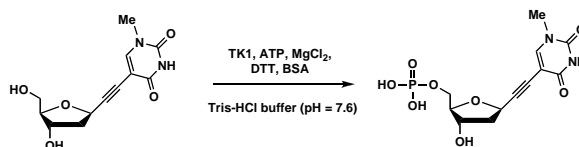
Scheme 6. Enzymatic synthesis of dT*MP from dT*.

Chemical Mono-Phosphorylation of T* Nucleoside
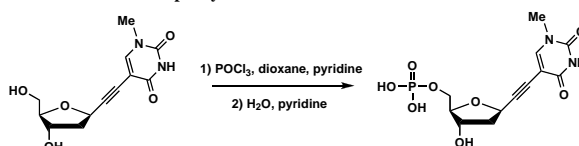
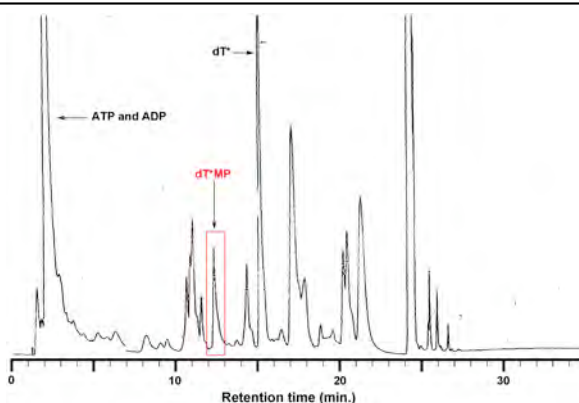**Scheme 7.** Chemical synthesis of dT*MP from dT*.

Figure 11. An HPLC chart for TK1 enzymatic reaction of T* nucleoside after 18 h.
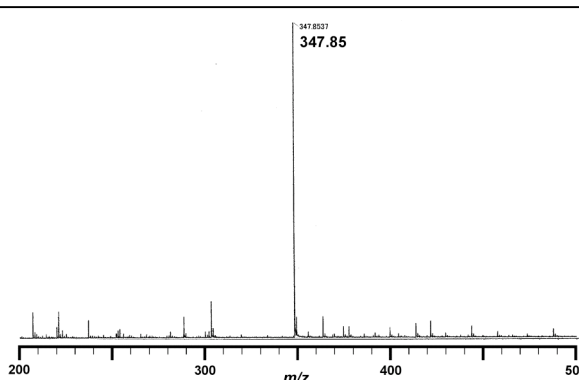

Figure 12. ESI mass spectrum (positive mode) of the preparative peak at 12.2 min in the HPLC analysis of the enzymatic reaction. Exact mass is 347.06 for [dT*MP + H$^+$]$^+$.

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1) Yasuhiro Doi, Junya Chiba, Tomoyuki Morikawa, <u>Masahiko Inouye</u>, "Artificial DNA Made Exclusively of Nonnatural Nucleosides with Four Types of Nonnatural Bases", ***J. Am. Chem. Soc.***, *130*, 8762–8768, **2008**.

2) <u>Masahiko Inouye</u>, Yasuhiro Doi, Junichi Azuchi. Wataru, Shirato, Junya Chiba, Hajime Abe, "Hexamethyldisilazane–Promoted Sonogashira Reaction of Polyfunctionalized *N*-Containing Heterocycles", ***Heterocycles***, *82*, 1137–1141, **2010**.

3) Masayoshi Takase, Tomoyuki Morikawa, Hajime Abe, <u>Masahiko Inouye</u>, "Stereoselective Synthesis of Alkynyl *C*-2-Deoxy-*β*-D-ribofuranosides via Intramolecular Nicholas Reaction: A Versatile Building Block for Nonnatural *C*-Nucleosides", ***Org. Lett.***, *5*, 625–628, **2003**.

4) Hajime Abe, Masayoshi Takase, Yasuhiro Doi, Shinya Matsumoto, Masaru Furusyo, <u>Masahiko Inouye</u>, "Tautomeric Self-Dimerization and Molecular Recognition Properties of 2-Aminopyrimidinone Derivatives as Triple Hydrogen-Bonding Modules in Molecular Assemblies", ***Eur. J. Org. Chem***., 2931–2940, **2005**.

List of Publications:

Publications
• <u>Masahiko Inouye</u>, Yasuhiro Doi, Junichi Azuchi. Wataru, Shirato, Junya Chiba, Hajime Abe, "Hexamethyldisilazane–Promoted Sonogashira Reaction of Polyfunctionalized *N*-Containing Heterocycles", ***Heterocycles***, *82*, 1137–1141, **2010**.

Conference presentations
• Junichi Azuchi, Junya Chiba, <u>Masahiko Inouye</u>, "Synthesis and oligomerization of alkynyl *C*-nucleosides with three types of artificial nucleobases", 6th Annual Meeting of Japanese Society of Chemical Biology, Tokyo, May 23–25 (2011): poster presentation.

• Wataru Shirato, Junya Chiba, <u>Masahiko Inouye</u>, "Evaluation of Thermodynamic Parameters for Heteroduplexes Consisting of Natural DNAs and Artificial DNAs Made of Alkynyl *C*-Nucleosides", 6th Annual Meeting of Japanese Society of Chemical Biology, Tokyo, May 23–25 (2011): poster presentation.

• Junichi Azuchi, Junya Chiba, <u>Masahiko Inouye</u>, "Synthesis and oligomerization of alkynyl *C*-nucleosides with a different linking position of the bases", The 91st Annual Meeting of The Chemical Society of Japan, Kanagawa, March 26–29 (2011): oral presentation.

• Wataru Shirato, Junya Chiba, <u>Masahiko Inouye</u>, "Investigation of the interaction between artificial DNA based on alkynyl *C*-nucleosides and natural DNA", The 91st Annual Meeting of The Chemical Society of Japan, Kanagawa, March 26–29 (2011): oral presentation.

• Junichi Azuchi, Junya Chiba, <u>Masahiko Inouye</u>, "Synthesis of novel alkynyl *C*-nucleosides possessing three types of artificial nucleobases", Annual Hokuriku Seminar of Organic Synthetic Chemistry in 2010, Ishikawa, October 8 (2010): poster presentation.